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(57) Abstract

The invention provides a competition binding assay for detecting $P2Y_{ADP}$ receptor ligands.

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NEW ASSAY

The present invention relates to an assay for detecting $P2Y_{ADP}$ receptor ligands.

It is known that ADP plays a pivotal role in terms of platelet function by causing adhesion, degranulation, shape change and aggregation of platelets via its interaction with cell surface P2 receptors. It is the $P2Y_{ADP}$ receptor (formerly known as P_{2T}) that is primarily involved in mediating platelet aggregation, which is an as yet uncloned G-protein linked receptor. The pharmacological characteristics of this receptor have been described, for example, in the references by Humphries et al., Br. J. Pharmacology, (1994), 113, 1057-1063, and Fagura et al., Br. J. Pharmacology, (1998), 124, 157-164.

Compounds having antagonist activity at the $P2Y_{ADP}$ receptor are known, for example, from WO 98/28300, WO 97/03084, WO 94/18216 and EP-A-508 687 and are useful as anti-thrombotic agents in the treatment or prophylaxis of diseases such as unstable angina, coronary angioplasty (PTCA) and myocardial infarction.

It would be desirable to identify further compounds with binding activity at the $P2Y_{ADP}$ receptor, and also to identify further tissues/cell lines containing this receptor.

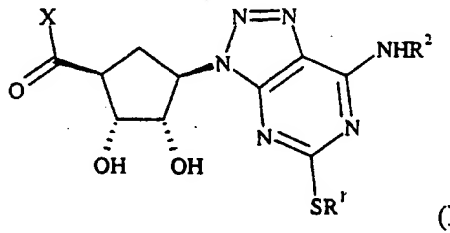
In accordance with the present invention, there is therefore provided a competition binding assay which comprises contacting a $P2Y_{ADP}$ receptor, preferably a human $P2Y_{ADP}$ receptor, with a $P2Y_{ADP}$ receptor radioligand and a candidate $P2Y_{ADP}$ receptor ligand, and measuring bound radioactivity.

The assay according to the present invention is very conveniently carried out on multi-well microtitre plates, thereby enabling a fast, simple and reproducible way of screening large numbers of potential $P2Y_{ADP}$ receptor ligands.

In the context of the present specification, the term "$P2Y_{ADP}$ receptor ligand", unless otherwise indicated, defines a ligand, e.g. an agonist or antagonist, of the $P2Y_{ADP}$ receptor other than a naturally-occurring ligand. The ligand may, for example, be a chemical compound, or a salt or solvate thereof.

The radioligand used in the assay is a substance which binds to the $P2Y_{ADP}$ receptor and which may be synthesised containing one or more radioactive atoms. Examples of substances which when radiolabelled may be used as the radioligand include:

(a) Compounds of formula (I)



(I)

wherein

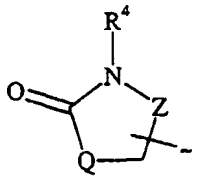
X is OH or NHR^3;

R^1 is C_{1-6}-alkyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more halogen atoms and/or OR^4, NR^4R^5, C_{1-6}-thioalkyl and/or C_{1-6}-alkyl (itself optionally substituted by one or more halogen atoms);

R^2 is C_{1-8}-alkyl or C_{2-8}-alkenyl each of which is optionally substituted by one or more halogen atoms and/or OR^4, NR^4R^5, C_{1-6}-thioalkyl, C_{3-8}-cycloalkyl, aryl and/or C_{1-6}-alkyl groups; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more halogen atoms and/or OR^4, NR^4R^5, C_{1-6}-thioalkyl, phenyl and/or C_{1-6}-alkyl groups; the optional phenyl substituent being further optionally substituted by one or more halogen atoms and/or NO_2, $C(O)R^4$, OR^4, NR^4R^5, C_{1-6}-thioalkyl and/or C_{1-6}-alkyl groups;

R^3 is hydrogen or C_{1-6}-alkyl substituted by one or more hydroxy and/or phenyl groups and optionally by one or more halogen atoms, wherein the phenyl group is substituted by one or more hydroxy groups and optionally substituted by one or more halogen atoms and/or NO_2, $C(O)R^4$, OR^4, NR^4R^5,C_{1-6}-thioalkyl and/or C_{1-6}-alkyl groups, or R^3 is a C_{1-6}-alkyl group substituted by a $C(O)NR^4R^5$ or a COOH group and optionally by one or more halogen atoms and/or OR^4, $C(NH)NR^4R^5$, $C(O)NR^4R^5$, phenyl and/or C_{1-6}-alkyl groups, wherein the alkyl group is optionally substituted by one or more hydroxy and/or phenyl groups and wherein the phenyl group is optionally substituted as defined above for R^3; or

R^3 is a lactam ring of formula (i):

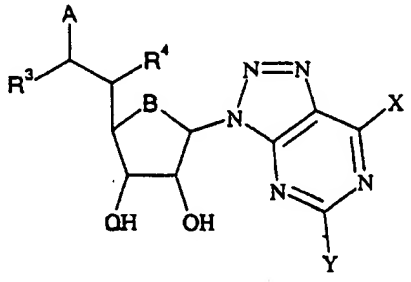


wherein Q is a $(CH_2)_m$ moiety wherein m is 1, 2 or 3, Z is O, C(O) or CH_2;

R^4 and R^5 each independently represent hydrogen, phenyl or a C_{1-6}-alkyl wherein the alkyl group is optionally substituted by one or more phenyl groups;

and salts and solvates thereof;

(b) Compounds of formula (II)



(II)

wherein

B is O or CH_2;

X is selected from NR^1R^2, SR^1 and C_1-C_7 alkyl;

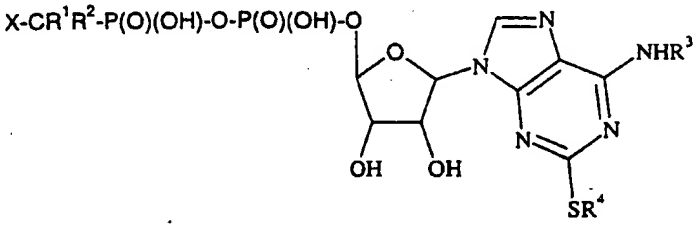
Y is selected from NR^1R^2, SR^1 and C_1-C_7 alkyl;

R^1 and R^2 is each and independently selected from H, or C_1-C_7 alkyl optionally substituted upon or within the alkyl chain by one or more of O, S, N or halogen;

R^3 and R^4 are both hydrogen, or R^3 and R^4 together form a bond;

A is COOH, $C(O)NH(CH_2)_pCOOH$, $C(O)N[(CH_2)_qCOOH]_2$, $C(O)NHCH(COOH)(CH_2)_rCOOH$ or 5-tetrazolyl, wherein p, q and r is each and independently 1, 2 or 3; and salts and solvates thereof;

(c) Compounds of formula (III)



(III)

wherein

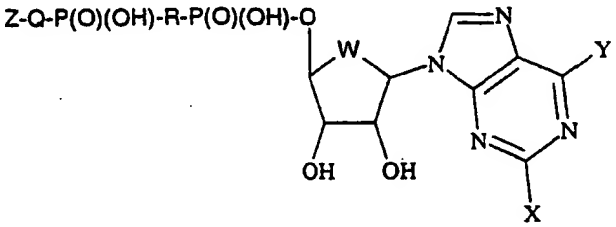
R^1 and R^2 independently represent hydrogen or halogen;

R^3 and R^4 independently represent phenyl, or C_1-C_6 alkyl optionally substituted by one or more substituents selected from OR^5, C_1-C_6 alkylthio, NR^6R^7, phenyl, $COOR^8$ and halogen;

R^5, R^6, R^7 and R^8 independently represent hydrogen or C_1-C_6 alkyl;

X represents an acidic moiety; and salts and solvates thereof;

(d) Compounds of formula (IV)



(IV)

wherein

Q represents CR^1R^2;

R represents O or CR^3R^4;

W represents O or CH_2;

R^1, R^2, R^3 and R^4 independently represent hydrogen or halogen;

X represents $S(O)_nR^5$, C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 acylamino, $CONR^6R^7$, NR^8R^9, halogen, a 5- or 6-membered S containing heterocycle, or phenyl optionally substituted by C_1-C_6 alkyl;

n represents 0, 1 or 2;

R^5 represents aryl or C_1-C_6 alkyl optionally substituted by one or more substituents selected from hydroxy, C_1-C_6 alkoxy, halogen and aryl;

R^6, R^7, R^8 and R^9 independently represent hydrogen or C_1-C_6 alkyl;

Y represents NH_2 or C_1-C_6 alkoxy;

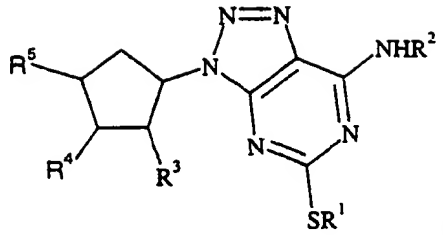
Z represents an acidic moiety;

in addition, when R represents CR^3R^4, then -Q-Z may also represent hydroxy or $-OP(O)(OH)_2$, provided that:

i) when R is O, W is O, X is Cl, Y is NH_2 and Z is $-P(O)(OH)_2$, then CR^1R^2 does not represent CH_2; and

ii) when R is O, W is O, X is SCH_3, Y is NH_2 and Z is $-P(O)(OH)_2$, then CR^1R^2 does not represent CH_2, CF_2 or CCl_2; and salts and solvates thereof;

(e) Compounds of formula (V)



(V)

wherein

R^1 is a C_{1-6} alkyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11} or C_{1-6} alkyl (itself optionally substituted by one or more halogen atoms);

R^2 is C_{1-8} alkyl optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11}, C_{3-8}-cycloalkyl, aryl (optionally substituted by one or more alkyl groups and/or halogen atoms), or C_{1-6}-alkyl; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11}, C_{1-6}-alkyl or phenyl (the latter two being optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^8$, OR^8, SR^{11}, $NR^{12}R^{13}$, phenyl and C_{1-6}-alkyl which is optionally substituted by one or more halogen atoms);

one of R^3 or R^4 is hydroxy and the other is hydrogen, hydroxy or NR^9R^{10};

R^5 is $(CH_2)_nNR^{14}R^{15}$ where n is 0 to 6 and R^{14} and R^{15} are independently hydrogen, C_{1-6}-alkyl or phenyl; or R^5 is $CONR^{16}R^{17}$ where R^{16} is hydrogen or C_{1-6}-alkyl, and R^{17} is C_{1-6}-alkyl or C_{3-6}-cycloalkyl each of which is substituted by $NR^{18}R^{19}$ and optionally substituted by phenyl, or R^{17} is C_{1-6}-alkyl or C_{3-6}-cycloalkyl substituted by phenyl which is substituted by $NR^{18}R^{19}$ where R^{18} and R^{19} are independently hydrogen, C_{1-6}-alkyl or phenyl; or R^{17} is a 5- to 8-membered saturated heterocycle containing one or more nitrogen atoms and optionally substituted on nitrogen by hydrogen, C_{1-6}-alkyl or phenyl;

or R^{16} and R^{17} together with the nitrogen atom to which they are attached form a 5- to

8-membered ring which is substituted by $NR^{18}R^{19}$ as defined above; or

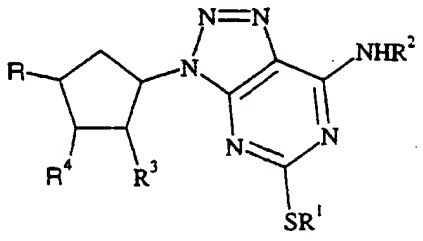
R^{16} together with R^{19} forms a 6- to 8-membered ring containing the two nitrogen atoms in which R^{17} and R^{18} are as defined above; or R^5 is $(CH_2)_pNR^{20}CO(CH_2)_qOR^{21}$ or $(CH_2)_pNR^{22}(CH_2)_qNR^{23}COR^{24}$ where p and q are independently 1 to 4 and R^{20}, R^{21}, R^{22}, R^{23} and R^{24} are independently C_{1-4}-alkyl or phenyl; or R^5 is $CH=CHCH_2NR^{25}R^{26}$ where R^{25} is hydrogen, C_{1-6} alkyl or phenyl and R^{26} is hydrogen or $(CH_2)_yNR^{27}R^{28}$ where y is 2 - 4 and R^{27} and R^{28} are independently hydrogen, C_{1-6} alkyl or phenyl;

R^8, R^9, R^{10} and R^{11} are independently hydrogen or C_{1-6}-alkyl; and

R^{12} and R^{13} are independently hydrogen, C_{1-6}-alkyl or acyl groups;

and salts and solvates thereof;

(f) Compounds of formula (VI)



(VI)

wherein

R^1 is a C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11} or C_{1-6} alkyl (itself optionally substituted by one or more halogen atoms);

R^2 is C_{1-8} alkyl optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11}, C_{3-8}-cycloalkyl, aryl (optionally substituted by one or more alkyl groups and/or halogen atoms), or C_{1-6}-alkyl; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11},

C_{1-6}-alkyl or phenyl, the latter two groups being optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^8$, OR^8, SR^{11}, $NR^{12}R^{13}$, a fused 5- or 6-membered saturated ring containing one or two oxygen atoms, phenyl or C_{1-6}-alkyl the latter two groups being optionally substituted by OR^8, NR^9R^{10} or one or more halogen atoms;

one of R^3 and R^4 is hydroxy and the other is hydrogen, hydroxy or NR^9R^{10};

R is a group $(CR^5R^6)_mOR^7$ where m is 0 or 1, R^5 and R^6 are independently hydrogen, C_{1-6} alkyl or phenyl the latter two groups being optionally substituted by halogen, and R^7 is hydrogen, C_{1-6} alkyl or $(CR^5R^6)_nR^{14}$ where R^5 and R^6 are as defined above, n is 1 to 3 and R^{14} is COOH, OR^{15}, $NR^{16}R^{17}$ or $CONR^{16}R^{17}$;

or R is a C_{1-4} alkyl or C_{2-4} alkenyl group, each of which is substituted by one or more groups selected from =S, =O, $=NR^{20}$ or OR^{21} and optionally substituted by one or more groups selected from halogen, C_{1-4} alkyl, phenyl, SR^{21}, NO_2 or $NR^{22}R^{23}$ (where R^{21}, R^{22} and R^{23} are independently hydrogen, C_{1-4} alkyl or phenyl; R^{20} is OR^{24} or $NR^{25}R^{26}$, where R^{24} is hydrogen, C_{1-4} alkyl or phenyl, and R^{25} and R^{26} are independently hydrogen, C_{1-4} alkyl, aryl, C_{1-6} acyl, arylsulphonyl or arylcarbonyl);

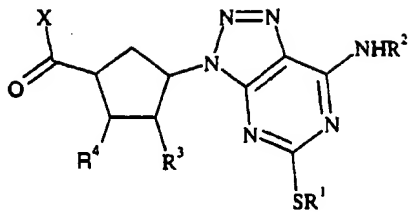
R^8 is hydrogen, C_{1-6} alkyl optionally substituted by halogen or R^8 is phenyl optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^6$, OR^6, SR^9, $NR^{10}R^{11}$;

R^9, R^{10} and R^{11} are independently hydrogen or C_{1-6} alkyl;

R^{12} and R^{13} are independently hydrogen, C_{1-6} alkyl, acyl, alkyl sulfonyl optionally substituted by halogen, or phenyl sulfonyl optionally substituted by C_1-C_4 alkyl; and

R^{15}, R^{16} and R^{17} are independently hydrogen or C_{1-6} alkyl; and salts and solvates thereof; and

(g) Compounds of formula (VII)



(VII)

wherein

R^1 is a C_{1-6} alkyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more substituents selected from halogen, OR^6, NR^7R^8, SR^9 or C_{1-6} alkyl (itself optionally substituted by one or more halogen atoms);

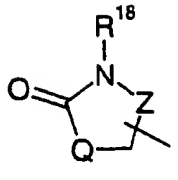
R^2 is C_{1-8} alkyl optionally substituted by one or more substituents selected from halogen, OR^6, NR^7R^8, SR^9, C_{3-8}-cycloalkyl, aryl (optionally substituted by one or more alkyl groups and/or halogen atoms), or C_{1-6}-alkyl; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more substituents selected from halogen, OR^6, NR^7R^8, SR^9, C_{1-6}-alkyl or phenyl (the latter two being optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^6$, OR^6, SR^9, $NR^{10}R^{11}$, phenyl and C_{1-6}-alkyl which is optionally substituted by one or more halogen atoms);

one of R^3 or R^4 is hydrogen and the other is hydroxy;

X is OH or NHR^5;

R^5 is a C_{1-6}-alkyl group substituted by COOH or $C(O)NR^7R^8$ and optionally by one or more further substituents selected from halogen, OR^{12}, $C(NH)NR^{13}R^{14}$, $C(O)NR^{15}R^{16}$, phenyl (optionally substituted by one or more groups selected from halogen, NO_2, $C(O)R^6$, OR^6, NR^7R^8, SR^9 and C_{1-6}-alkyl) or C_{1-6}-alkyl (optionally substituted by one or more hydroxy or phenyl groups);

or R^5 is a lactam ring of formula (i):



(i)

wherein Q is a $(CH_2)_m$ moiety where m is 1, 2 or 3, Z is O, C(O) or CH_2 and R^{18} is hydrogen or C_{1-6}-alkyl;

R^6, R^9, R^{12}, R^{13}, R^{14}, R^{15} and R^{16} are independently hydrogen or C_{1-6}-alkyl;

R^7 and R^8 are independently hydrogen, C_{1-6}-alkyl (optionally substituted by one or more phenyl groups) or phenyl groups; and

R^{10} and R^{11} are independently hydrogen, C_{1-6}-alkyl or acyl groups;

and salts and solvates thereof.

Compounds of formulae (I), (II), (III), (IV), (V), (VI) and (VII) are disclosed respectively in WO 98/28300, WO 97/03084, WO 94/18216, EP-A-508 687, PCT/SE98/01392, PCT/SE98/01393 and PCT/SE98/01394 and the contents of these seven documents are incorporated herein by reference.

Examples of suitable salts that may be used include alkali metal (e.g. sodium or potassium), alkaline earth metal (e.g. calcium or magnesium), Group III metal (e.g. aluminium), ammonium, hydrochloride, hydrobromide, phosphate, acetate, fumarate, maleate, tartrate, citrate, oxalate, methanesulphonate and *p*-toluenesulphonate salts.

The techniques for radiolabelling substances may be those conventionally used in the art and therefore the radioligand may be prepared by methods known in the art.

The radioligand is most preferably a radiolabelled compound of formula (I) or(II) or a salt or solvate thereof, and is especially [^{125}I]-[1*S*-[1α,2β,3β,4α(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid, or [^{3}*H*]-

[1*S*-(1α,2β,3β,4α)]-4-[7-(butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]- 2,3-dihydroxy-cyclopentanepropanoic acid, or a salt or solvate of any one thereof.

Advantageously, the radioligand has a specific activity of greater than 10 Ci/mmol and a $P2Y_{ADP}$ receptor activity (IC_{50}) of less than 1 micromolar (μM).

In the context of the present specification, "specific activity" is defined as the activity per unit mass of substance containing a radioactive nuclide and is normally expressed as millicuries per milligram (mCi/mg) (kBq/mg), as millicuries per millimole (mCi/mmol) (kBq/mmol), or as curies per millimole (Ci/mmol) (GBq/mmol); and "$P2Y_{ADP}$ receptor activity (IC_{50})" is defined as the concentration, expressed in micromolar units, of radioligand required to inhibit the maximal aggregation response elicited by ADP according to the platelet aggregation assay as described in WO 98/28300. The platelet aggregation assay, which uses washed human platelets, is carried out in the following manner.

Human venous blood (100 ml) is divided equally between 3 tubes, each containing 3.2% trisodium citrate (4 ml) as anti-coagulant. The tubes are centrifuged for 15 minutes at 240G to obtain a platelet-rich plasma (PRP) to which 300 ng/ml prostacyclin is added to stabilize the platelets during the washing procedure. Red cell free PRP is obtained by centrifugation for 10 minutes at 125G followed by further centrifugation for 15 minutes at 640G. The supernatant is discarded and the platelet pellet resuspended in modified, Calcium Free Tyrode solution (10 ml) (CFT), composition: NaCl 137mM, $NaHCO_3$ 11.9mM, NaH_2PO_4 0.4mM, KCl 2.7 mM, $MgCl_2$ 1.1 mM, dextrose 5.6 mM, gassed with 95% O_2/5% CO_2 and maintained at 37°C. Following addition of a further 300 ng/ml PGI_2, the pooled suspension is centrifuged once more for 15 minutes at 640G. The supernatant is discarded and the platelets are resuspended initially in 10 ml CFT with further CFT added to adjust the final platelet count to $2x10^5$/ml. This final suspension is stored in a 60 ml syringe at 3°C with air

excluded. To allow recovery from PGI_2-inhibition of normal function, platelets are used in aggregation studies no sooner than 2 hours after final resuspension.

Aliquots of platelet suspension (3 ml) are added to tubes containing $CaCl_2$ solution (60 µl of 50 mM solution with a final concentration of 1mM). Human fibrinogen (Sigma, F 4883) and 8-sulphophenyltheophylline (8-SPT, which is used to block any P_1-agonist activity of test substance) are added to give final concentrations of 0.2 mg/ml (60 µl of 10 mg/ml solution of clottable protein in saline) and 300 nM (10 µl of 15 mM solution in 6% glucose), respectively. Platelets or buffer as appropriate are added in a volume of 150 µl to the individual wells of a 96 well plate. All measurements are made in triplicate in platelets from each donor.

Aggregation responses in 96 well plates are measured using the change in absorbance given by the plate reader at 660 nm. Either a Bio-Tec Ceres 900C or a Dynatech MRX are used as the plate reader.

The absorbance of each well in the plate is read at 660 nm to establish a baseline figure. Saline or the appropriate solution of test substance (e.g. the radioligand) is added to each well in a volume of 10 µl to give a final concentration of 0, 0.01, 0.1, 1, 10 or 100 mM. The plate is then shaken for 5 minutes on an orbital shaker on setting 10 and the absorbance read at 660 nm. Aggregation at this point is indicative of agonist activity of the test substance. Saline or ADP (30 mM; 10 µl of 450 mM) is then added to each well and the plate shaken for a further 5 minutes before reading the absorbance again at 660 nm. The concentration of test substance that produces a response which is half the maximum control ADP response is the IC_{50} value.

According to a preferred embodiment of the invention, the competition binding assay comprises

(i) isolating and washing human platelets or human platelet membranes,

(ii) incubating the platelets or platelet membranes with a $P2Y_{ADP}$ receptor radioligand and a candidate $P2Y_{ADP}$ receptor ligand,

(iii) filtering and washing the platelets or platelet membranes, and

(iv) measuring bound radioactivity.

In step (i) above, methods for isolating and washing human platelets or human platelet membranes are known in the art, e.g. as described by Connolly et al. (1992), J. Biol. Chem., 267, 6893-6898 and Biochim. et Biophys. Act. (1986), 854, 67-76.

In step (ii), the incubation is conveniently carried at a temperature in the range from 4 to 37°C, for a period of time of from 5 to 120 minutes.

The present invention further provides the use of [^{125}I]-[1*S*-[1α,2β,3β,4α(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid, or [^{3}*H*]-[1*S*-(1α,2β,3β,4α)]-4-[7-(butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]-2,3-dihydroxy-cyclopentanepropanoic acid, or a salt or solvate of any one thereof, as a $P2Y_{ADP}$ receptor radioligand in a competition binding assay as hereinbefore defined.

The present invention will now be further illustrated by reference to the following Examples.

Example 1

Synthesis of [^{3}*H*]-[1*S*-(1α,2β,3β,4α)]-4-[7-(Butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]-2,3-dihydroxy-cyclopentanepropanoic acid, sodium salt

A flask containing [1*R*-[1α(*E*),2β,3β,4α]]-3-[4-[7-(butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-2,3-dihydroxycyclopentyl]-2-propenoic acid (5.5mg) (prepared as described in Example 3 of WO 97/03084) in ethanol (1ml),

containing palladium on carbon (5% w/w Pd, 1mg) was attached to a tritium manifold, evacuated and tritium gas (10 Ci, 57.6 Ci $mmol^{-1}$, 174 μmol) introduced. The reaction was stirred for 18 hours at room temperature, then the catalyst removed by filtration and the remaining tritium removed by lyophilisation with ethanol (2x1ml). Purification (HPLC, Symmetry C8, 45% acetonitrile/0.025% v/v aqueous acetic acid as eluant) gave the title acid which was converted to the sodium salt. The salt was dissolved in ethanol (16ml) to afford a solution of the title compound (1 mCi ml^{-1}, 24 Ci $mmol^{-1}$). The product was characterised by comparison with unlabelled compound (prepared as described in Example 3 of WO 97/03084) (HPLC, Symmetry C8, 50% methanol/0.1% w/v aqueous ammonium acetate to 95% methanol over 10 minutes as eluant).

Example 2

Synthesis of [^{125}I]- [1*S*-[1α,2β,3β,4α(*E*)]]-2,3-Dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid

[3a*R*-[3aα,4α,6α(*E*),6aα]]-6-[7-(3-Tributylstannyl-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-tetrahydro-2,2-dimethyl-4*H*-cyclopenta-1,3-dioxole-4-carboxylic acid (1.66 nmol) (prepared as described in Example 93 of WO 98/28300) in acetonitrile (40μl) was added to sodium [^{125}I]iodide (obtained from Amersham International) (1 mCi, 10 μl, 0.5 nmol), followed by a solution of chloramine-T in water (7 μl, 1.5 nmol). The vial was sealed, shaken vigorously, then left to stand at room temperature for 1 hour. Aqueous trifluoroacetic acid (25% v/v, 50 μl) was then added and the reaction mixture resealed, shaken and left to stand for a further 2 hours. Purification (HPLC, Novapak C18, 30% acetonitrile/0.5% w/v aqueous ammonium acetate then increased to 95% acetonitrile as eluant) gave the title compound, to which ethanol was added to the required volume. The product was characterised by comparison with unlabelled compound (prepared as described in Example 93 of WO 98/28300) (HPLC symmetry shield C8 75 x 3.9 mm, 25% acetonitrile/0.5% w/v aqueous ammonium acetate increased to 95% acetonitrile over 3 minutes and held for 3 minutes, 2 ml min^{-1}, retention time = 2.98 minutes).

Example 3

WASHED PLATELET PREPARATION

Human venous blood (100 ml), obtained from healthy volunteers was divided equally between 3 tubes, each containing 3.2% trisodium citrate (4ml) as an anti-coagulant. The tubes were centrifuged for 15 min at 240G to obtain platelet-rich plasma (PRP) to which prostacyclin (PGI_2 300 $ng \cdot ml^{-1}$) was added to stabilize platelets during the washing procedure. Red cell-free PRP was obtained by centrifugation for 10 minutes at 125G and following further centrifugation for 15 minutes at 640G, the supernatant was discarded and the platelet pellet in each tube resuspended in modified, calcium-free, Tyrodes solution (10ml) (CFT, composition: NaCl, 137.0 mM; $NaHCO_3$, 11.9 mM; NaH_2PO_4, 0.4 mM; KCl, 2.7 mM; $MgCl_2$, 1.1 mM and dextrose, 5.55 mM), gassed with 95% O_2/5% CO_2 and maintained at 37°C. Following addition of PGI_2 (300 $ng \cdot ml^{-1}$), the pooled suspension was centrifuged once more for 15 minutes at 640G. The supernatant was discarded and the platelets resuspended in CFT to give a final platelet count of 200-250 x $10^3 \cdot \mu l^{-1}$. The platelets were used within 30 min for radioligand binding studies.

Example 4

BINDING ASSAYS

Binding assays were performed in 96-well plates, with each well containing 250 µl aliquots of CFT consisting of 50 µl 0.1 µCi [^{125}I]- [1*S*-[1α,2β,3β,4α(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid (final concentration 0.18 nM) or 50 µl 0.1 µCi [^{3}*H*]-[1*S*-(1α,2β,3β,4α)]-4-[7-(Butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]-2,3-dihydroxy-cyclopentanepropanoic acid, sodium salt (final concentration 17 nM) and 200 µl of washed platelets at a concentration of 200-250 x $10^3 \cdot \mu l^{-1}$ (final concentration 160-200 x $10^3 \cdot \mu l^{-1}$). All putative $P2Y_{ADP}$ ligands were tested in duplicate over the appropriate concentration range by addition of 5 µl of compound prior to adding the radioligand, with appropriate solvent controls being performed in parallel. The plates were

incubated for 30 min at room temperature on a plate shaker (Stuart scientific; model S01, setting 6) prior to terminating the reactions by filtration. Filtration was performed using a MACHIII cell harvester with 2 x 2s wash periods (with CFT) on to Whatman GF/B filter plates for platelets incubated [^{125}I]- [1*S*-[1α,2β,3β,4α(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid or a Wallac cell harvester using glass fibre printed filtermats type A, with a 7s wash time (with CFT) for platelets incubated with [^{3}*H*]-[1*S*-(1α,2β,3β,4α)]-4-[7-(Butylamino)-5-(propylthio)-3*H*1,2,3-triazolo[4,5-*d*]-pyrimidin-3-yl]-2,3-dihydroxy-cyclopentanepropanoic acid, sodium salt. The resultant filterplates were then, in the case of the MACHIII, sealed and Microscint 20 (50 µl) added prior to determination of [^{125}I] levels by scintillation counting on a Packard Topcounter or, in the case of the Wallac filtermats, the individual wells were punched into vials containing 5 ml scintillation fluid for determination of [^{3}H] levels in a beta counter.

Non-specific binding was determined in the presence of the standard $P2Y_{ADP}$ antagonist, 2-propylthio-D-β,γ-dichloromethylene ATP (10 µM), as described by Humphries et al., Br. J. Pharmacology (1995), 115, 1110-1116.

Results were expressed as specific binding in CPM/DPM and were calculated by subtracting the non-specific binding from the total binding achieved at each concentration. For each test compound, a binding affinity (IC_{50}) was calculated by linear interpolation of the concentration/inhibition curve, using the software package Excel. The IC_{50} value being the concentration at which a 50% reduction in specific binding of either [^{125}I]-[1*S*-[1α,2β,3β,4α(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid or [^{3}*H*]-[1*S*-(1α,2β,3β,4α)]-4-[7-(butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]-2,3-dihydroxy-cyclopentanepropanoic acid, sodium salt was achieved. Results were reported as pKi values which are equal to the negative logarithm of the IC_{50} (pIC_{50}) in this system (Cheng Prusoff). In cases where there was insufficient displacement of binding to calculate a pKi value, the activities were reported as being < 6.

CLAIMS

1. A competition binding assay, which comprises contacting a $P2Y_{ADP}$ receptor with a $P2Y_{ADP}$ receptor radioligand and a candidate $P2Y_{ADP}$ receptor ligand, and measuring bound radioactivity.

2. An assay according to claim 1, wherein the $P2Y_{ADP}$ receptor is of human origin.

3. An assay according to claim 2 which comprises

(i) isolating and washing human platelets or human platelet membranes,

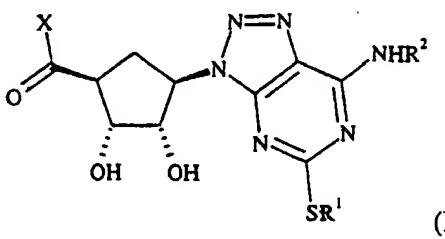
(ii) incubating the platelets or platelet membranes with a $P2Y_{ADP}$ receptor radioligand and a candidate $P2Y_{ADP}$ receptor ligand,

(iii) filtering and washing the platelets or platelet membranes, and

(iv) measuring bound radioactivity.

4. An assay according to any one of claims 1 to 3, wherein the radioligand has a specific activity greater than 10 Ci/mmol and a $P2Y_{ADP}$ receptor activity (IC_{50}) of less than 1 micromolar (μM).

5. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (I)



(I)

wherein

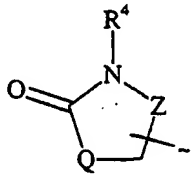
X is OH or NHR^3;

R^1 is C_{1-6}-alkyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more halogen atoms and/or OR^4, NR^4R^5,C_{1-6}-thioalkyl and/or C_{1-6}-alkyl (itself optionally substituted by one or more halogen atoms);

R^2 is C_{1-8}-alkyl or C_{2-8}-alkenyl each of which is optionally substituted by one or more halogen atoms and/or OR^4, NR^4R^5, C_{1-6}-thioalkyl, C_{3-8}-cycloalkyl, aryl and/or C_{1-6}-alkyl groups; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more halogen atoms and/or OR^4, NR^4R^5, C_{1-6}-thioalkyl, phenyl and/or C_{1-6}-alkyl groups; the optional phenyl substituent being further optionally substituted by one or more halogen atoms and/or NO_2, $C(O)R^4$, OR^4, NR^4R^5, C_{1-6}-thioalkyl and/or C_{1-6}-alkyl groups;

R^3 is hydrogen or C_{1-6}-alkyl substituted by one or more hydroxy and/or phenyl groups and optionally by one or more halogen atoms, wherein the phenyl group is substituted by one or more hydroxy groups and optionally substituted by one or more halogen atoms and/or NO_2, $C(O)R^4$, OR^4, NR^4R^5,C_{1-6}-thioalkyl and/or C_{1-6}-alkyl groups, or R^3 is a C_{1-6}-alkyl group substituted by a $C(O)NR^4R^5$ or a COOH group and optionally by one or more halogen atoms and/or OR^4, $C(NH)NR^4R^5$, $C(O)NR^4R^5$, phenyl and/or C_{1-6}-alkyl groups, wherein the alkyl group is optionally substituted by one or more hydroxy and/or phenyl groups and wherein the phenyl group is optionally substituted as defined above for R^3; or

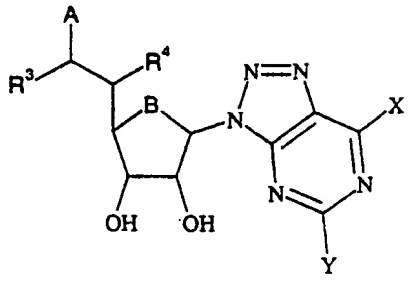
R^3 is a lactam ring of formula (i):



wherein Q is a $(CH_2)_m$ moiety wherein m is 1, 2 or 3, Z is O, C(O) or CH_2;

R^4 and R^5 each independently represent hydrogen, phenyl or a C_{1-6}-alkyl wherein the alkyl group is optionally substituted by one or more phenyl groups; or a salt or solvate thereof.

6. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (II)



(II)

wherein

B is O or CH_2;

X is selected from NR^1R^2, SR^1 and C_1-C_7 alkyl;

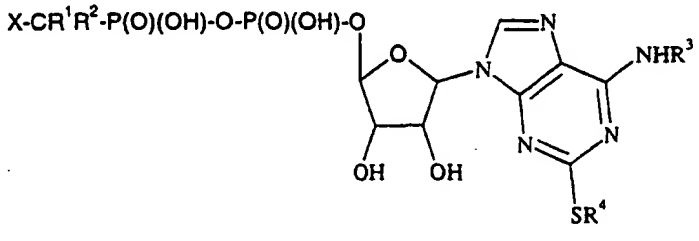
Y is selected from NR^1R^2, SR^1 and C_1-C_7 alkyl;

R^1 and R^2 is each and independently selected from H, or C_1-C_7 alkyl optionally substituted upon or within the alkyl chain by one or more of O, S, N or halogen;

R^3 and R^4 are both hydrogen, or R^3 and R^4 together form a bond;

A is COOH, $C(O)NH(CH_2)_pCOOH$, $C(O)N[(CH_2)_qCOOH]_2$, $C(O)NHCH(COOH)(CH_2)_rCOOH$ or 5-tetrazolyl, wherein p, q and r is each and independently 1, 2 or 3; or a salt or solvate thereof.

7. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (III)



(III)

wherein

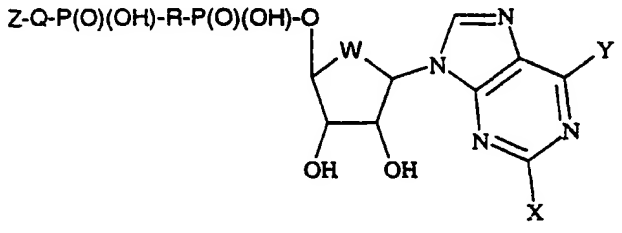
R^1 and R^2 independently represent hydrogen or halogen;

R^3 and R^4 independently represent phenyl, or C_1-C_6 alkyl optionally substituted by one or more substituents selected from OR^5, C_1-C_6 alkylthio, NR^6R^7, phenyl, $COOR^8$ and halogen;

R^5, R^6, R^7 and R^8 independently represent hydrogen or C_1-C_6 alkyl;

X represents an acidic moiety; or a salt or solvate thereof.

8. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (IV)



(IV)

wherein

Q represents CR^1R^2;

R represents O or CR^3R^4;

W represents O or CH_2;

R^1, R^2, R^3 and R^4 independently represent hydrogen or halogen;

X represents $S(O)_nR^5$, C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 acylamino, $CONR^6R^7$, NR^8R^9, halogen, a 5- or 6-membered S containing heterocycle, or phenyl optionally substituted by C_1-C_6 alkyl;

n represents 0, 1 or 2;

R^5 represents aryl or C_1-C_6 alkyl optionally substituted by one or more substituents selected from hydroxy, C_1-C_6 alkoxy, halogen and aryl;

R^6, R^7, R^8 and R^9 independently represent hydrogen or C_1-C_6 alkyl;

Y represents NH_2 or C_1-C_6 alkoxy;

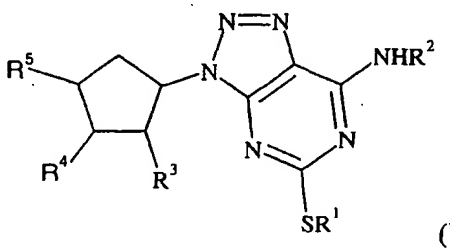
Z represents an acidic moiety;

in addition, when R represents CR^3R^4, then -Q-Z may also represent hydroxy or -$OP(O)(OH)_2$, provided that:

i) when R is O, W is O, X is Cl, Y is NH_2 and Z is -$P(O)(OH)_2$, then CR^1R^2 does not represent CH_2; and

ii) when R is O, W is O, X is SCH_3, Y is NH_2 and Z is -$P(O)(OH)_2$, then CR^1R^2 does not represent CH_2, CF_2 or CCl_2; or a salt or solvate thereof.

9. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (V)



(V)

wherein

R^1 is a C_{1-6} alkyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11} or C_{1-6} alkyl (itself optionally substituted by one or more halogen atoms);

R^2 is C_{1-8} alkyl optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11}, C_{3-8}-cycloalkyl, aryl (optionally substituted by one or more alkyl groups and/or halogen atoms), or C_{1-6}-alkyl; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11},

C_{1-6}-alkyl or phenyl (the latter two being optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^8$, OR^8, SR^{11}, $NR^{12}R^{13}$, phenyl and C_{1-6}-alkyl which is optionally substituted by one or more halogen atoms);

one of R^3 or R^4 is hydroxy and the other is hydrogen, hydroxy or NR^9R^{10};

R^5 is $(CH_2)_nNR^{14}R^{15}$ where n is 0 to 6 and R^{14} and R^{15} are independently hydrogen, C_{1-6}-alkyl or phenyl; or R^5 is $CONR^{16}R^{17}$ where R^{16} is hydrogen or C_{1-6}-alkyl, and R^{17} is C_{1-6}-alkyl or C_{3-6}-cycloalkyl each of which is substituted by $NR^{18}R^{19}$ and optionally substituted by phenyl, or R^{17} is C_{1-6}-alkyl or C_{3-6}-cycloalkyl substituted by phenyl which is substituted by $NR^{18}R^{19}$ where R^{18} and R^{19} are independently hydrogen, C_{1-6}-alkyl or phenyl; or R^{17} is a 5- to 8-membered saturated heterocycle containing one or more nitrogen atoms and optionally substituted on nitrogen by hydrogen, C_{1-6}-alkyl or phenyl;

or R^{16} and R^{17} together with the nitrogen atom to which they are attached form a 5- to 8-membered ring which is substituted by $NR^{18}R^{19}$ as defined above; or

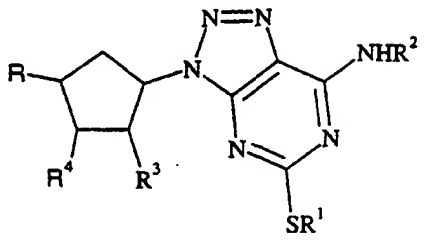
R^{16} together with R^{19} forms a 6- to 8-membered ring containing the two nitrogen atoms in which R^{17} and R^{18} are as defined above; or R^5 is $(CH_2)_pNR^{20}CO(CH_2)_qOR^{21}$ or $(CH_2)_pNR^{22}(CH_2)_qNR^{23}COR^{24}$ where p and q are independently 1 to 4 and R^{20}, R^{21}, R^{22}, R^{23} and R^{24} are independently C_{1-4}-alkyl or phenyl; or R^5 is $CH{=}CHCH_2NR^{25}R^{26}$ where R^{25} is hydrogen, C_{1-6} alkyl or phenyl and R^{26} is hydrogen or $(CH_2)_yNR^{27}R^{28}$ where y is 2 - 4 and R^{27} and R^{28} are independently hydrogen, C_{1-6} alkyl or phenyl;

R^8, R^9, R^{10} and R^{11} are independently hydrogen or C_{1-6}-alkyl; and

R^{12} and R^{13} are independently hydrogen, C_{1-6}-alkyl or acyl groups;

or a salt or solvate thereof.

10. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (VI)



(VI)

wherein

R^1 is a C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11} or C_{1-6} alkyl (itself optionally substituted by one or more halogen atoms);

R^2 is C_{1-8} alkyl optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11}, C_{3-8}-cycloalkyl, aryl (optionally substituted by one or more alkyl groups and/or halogen atoms), or C_{1-6}-alkyl; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more substituents selected from halogen, OR^8, NR^9R^{10}, SR^{11}, C_{1-6}-alkyl or phenyl, the latter two groups being optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^8$, OR^8, SR^{11}, $NR^{12}R^{13}$, a fused 5- or 6-membered saturated ring containing one or two oxygen atoms, phenyl or C_{1-6}-alkyl the latter two groups being optionally substituted by OR^8, NR^9R^{10} or one or more halogen atoms;

one of R^3 and R^4 is hydroxy and the other is hydrogen, hydroxy or NR^9R^{10};

R is a group $(CR^5R^6)_mOR^7$ where m is 0 or 1, R^5 and R^6 are independently hydrogen,

C_{1-6} alkyl or phenyl the latter two groups being optionally substituted by halogen, and R^7 is hydrogen, C_{1-6} alkyl or $(CR^5R^6)_nR^{14}$ where R^5 and R^6 are as defined above, n is 1 to 3 and R^{14} is COOH, OR^{15}, $NR^{16}R^{17}$ or $CONR^{16}R^{17}$;

or R is a C_{1-4} alkyl or C_{2-4} alkenyl group, each of which is substituted by one or more groups selected from =S, =O, $=NR^{20}$ or OR^{21} and optionally substituted by one or more groups selected from halogen, C_{1-4} alkyl, phenyl, SR^{21}, NO_2 or $NR^{22}R^{23}$ (where R^{21}, R^{22} and R^{23} are independently hydrogen, C_{1-4} alkyl or phenyl; R^{20} is OR^{24} or $NR^{25}R^{26}$, where R^{24} is hydrogen, C_{1-4} alkyl or phenyl, and R^{25} and R^{26} are independently hydrogen, C_{1-4} alkyl, aryl, C_{1-6} acyl, arylsulphonyl or arylcarbonyl);

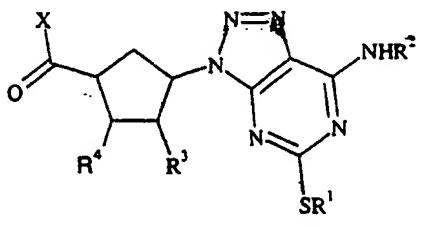
R^8 is hydrogen, C_{1-6} alkyl optionally substituted by halogen or R^8 is phenyl optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^6$, OR^6, SR^9, $NR^{10}R^{11}$;

R^9, R^{10} and R^{11} are independently hydrogen or C_{1-6} alkyl;

R^{12} and R^{13} are independently hydrogen, C_{1-6} alkyl, acyl, alkyl sulfonyl optionally substituted by halogen, or phenyl sulfonyl optionally substituted by C_1-C_4 alkyl; and

R^{15}, R^{16} and R^{17} are independently hydrogen or C_{1-6} alkyl; or a salt or solvate thereof.

11. An assay according to any one of claims 1 to 4, wherein the radioligand is a radiolabelled compound of general formula (VII)



(VII)

wherein

R^1 is a C_{1-6} alkyl, C_{3-8}-cycloalkyl or a phenyl group, each group being optionally substituted by one or more substituents selected from halogen, OR^6, NR^7R^8, SR^9 or C_{1-6} alkyl (itself optionally substituted by one or more halogen atoms);

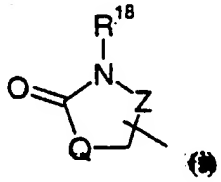
R^2 is C_{1-8} alkyl optionally substituted by one or more substituents selected from halogen, OR^6, NR^7R^8, SR^9, C_{3-8}-cycloalkyl, aryl (optionally substituted by one or more alkyl groups and/or halogen atoms), or C_{1-6}-alkyl; or R^2 is a C_{3-8}-cycloalkyl group optionally substituted by one or more substituents selected from halogen, OR^6, NR^7R^8, SR^9, C_{1-6}-alkyl or phenyl (the latter two being optionally substituted by one or more substituents selected from halogen, NO_2, $C(O)R^6$, OR^6, SR^9, $NR^{10}R^{11}$, phenyl and C_{1-6}-alkyl which is optionally substituted by one or more halogen atoms);

one of R^3 or R^4 is hydrogen and the other is hydroxy;

X is OH or NHR^5;

R^5 is a C_{1-6}-alkyl group substituted by COOH or $C(O)NR^7R^8$ and optionally by one or more further substituents selected from halogen, OR^{12}, $C(NH)NR^{13}R^{14}$, $C(O)NR^{15}R^{16}$, phenyl (optionally substituted by one or more groups selected from halogen, NO_2, $C(O)R^6$, OR^6, NR^7R^8, SR^9 and C_{1-6}-alkyl) or C_{1-6}-alkyl (optionally substituted by one or more hydroxy or phenyl groups);

or R^5 is a lactam ring of formula (i):



(i)

wherein Q is a $(CH_2)_m$ moiety where m is 1, 2 or 3, Z is O, C(O) or CH_2 and R^{18} is hydrogen or C_{1-6}-alkyl;

R^6, R^9, R^{12}, R^{13}, R^{14}, R^{15} and R^{16} are independently hydrogen or C_{1-6}-alkyl;

R^7 and R^8 are independently hydrogen, C_{1-6}-alkyl (optionally substituted by one or more phenyl groups) or phenyl groups; and

R^{10} and R^{11} are independently hydrogen, C_{1-6}-alkyl or acyl groups;

or a salt or solvate thereof.

12. An assay according to any one of claims 1 to 4, wherein the radioligand is selected from [^{125}I]-[1*S*-[1a,2b,3b,4a(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid, [^{3}*H*]-[1*S*-(1α,2β,3β,4α)] -4-[7-(butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]- 2,3-dihydroxy-cyclopentanepropanoic acid, and salts and solvates thereof.

13. An assay according to any one of the preceding claims, wherein the candidate $P2Y_{ADP}$ receptor ligand is a $P2Y_{ADP}$ receptor antagonist.

14. Use of [^{125}I]-[1*S*-[1a,2b,3b,4a(*E*)]]-2,3-dihydroxy-4-[7-(3-iodo-prop-2-enylamino)-5-(propylthio)-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidin-3-yl]-cyclopentanecarboxylic acid, or [^{3}*H*]-[1*S*-(1α,2β,3β,4α)] -4-[7-(butylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidin-3-yl]- 2,3-dihydroxy-cyclopentanepropanoic acid, or a salt or solvate of any one thereof, as a $P2Y_{ADP}$ receptor radioligand in a competition binding assay as defined in claim 1.

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(54) Title: NEW ASSAY

(57) Abstract

The invention provides a competition binding assay for detecting $P2Y_{ADP}$ receptor ligands.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/02252

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/566, C07D 487/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9629345 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA), 26 Sept 1996 (26.09.96), see example 3 and 4	1,2
Y		3,4
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X	US 5620676 A (KENNETH A. JACOBSON ET AL), 15 April 1997 (15.04.97), see example 10	1,2
	--	
X	WO 9719170 A1 (EUROSCREEN S.A.), 29 May 1997 (29.05.97), see pages 2 and 7-9 and claims 58-64	1,2
Y		3,4
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	05 -06- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/EÖ Telephone No. + 46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Dialog Information Services, File Medline Dialog accession no. 98303756, Hechler B. et al: "The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation", Blood 1998 Jul 1;92 (1):152-9	3
T	Dialog Information Services, File Medline, Dialog accession no. 99059677, Kunapuli SP et al: "P2 receptor subtypes in the cardiovascular system", Biochem J 1998 Dec 15;336 (Pt 3):513-23	1-3
A	Chemical Abstracts, Volume 128 (), (Columbus, Ohio, USA), Jin, Jianguo et al, "Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets", THE ABSTRACT No 203373, J. Biol. Chem. 1998, 273 (4), 2030-2034	1
X,Y	Chemical Abstracts, Volume 127 (), (Columbus, Ohio, USA), Puri, Rajinder N. et al, "Modulation of platelet responses by 2-(3-(Bromo-2-oxopropylthio)adenosine-5'-diphosphate involves its binding to as well as covalent modification of an ADP-aggregin", THE ABSTRACT No 133782, Arch. Biochem. Biophys. 1997, 343 (1), 140-145	3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE99/02252

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5-14

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE99/02252

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The prior art for $P2Y_{ADP}$ receptor ligand detection is revealed by e.g. WO9629345, WO9719170 and US5620676. Consequently and in the absence of a common inventive concept, the present invention relates to at least 8 such groups of inventions:

1 A competition binding assay, according to claims 1-4 and 12-14.

2-8 Seven different radioligand assays each characterised by the respective different radioligand defined in claims 5-14.

No novel special technical feature common to inventions 1-8, as required by Rule 13.2, has been found. Invention 1 has been searched.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 99/02252

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9629345 A1	26/09/96	US 5712258 A	27/01/98
US 5620676 A	15/04/97	NONE	
WO 9719170 A1	29/05/97	CA 2235627 A	29/05/97
		EP 0862628 A	09/09/98